Antitumor Activity Of Chronic Heart Failure Drug Quinolinone Derivate-Vesnarinone On An Oral Malignant Burkitt's Lymphoma Cell (Study on proliferation, chemotactic migration and cell apoptosis)

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Abstract

Vesnarinoneis known to have multifunctional biological activities including cell growth and metastases suppression of various types of human cancers. Vesnarinone has been widely used for the treatment of chronic heart failure because it's activity as an oral inotropic agent. In the present study, the antitumor activity of vesnarinone against oral malignant Burkitt lymphoma (Raji) cells through analysis of proliferative barriers, migratory chemotactic suppression and cell apoptosisinduction was examined. The pure laboratory experimental with post-test design only control group design was performed in this study. Raji cells were cultured until the 5th passage. Raji cellswere incubated with vesnarinone doses of 0, 1.25x10-2, 2.5x10-2 and 5x10-2 M, and IC_{50} carboplatin (3.1x 10-6 M) as positive control. After 24 and 48 hours incubation, cells were harvested in the Petry dish. Cell growth inhibition test or MTT assay was carried-out by ELISA reader. Cell migratory chemotaxis analysis was delivered by Boyden chamber kit, and cell apoptosis assay was detectedby double staining Acridine orange-Ethidium bromide (AO-EB).The results revealed quinolinone derivate-vesnarinone was significantly inhibited the cell proliferation and migratory chemotactic activity of an oral malignant Burkitt'slymphoma cells characterized by increased in the number of cells apoptosis (P<0.001). Elevated their inhibition and induction of cell apoptosis was proportional to an increase in vesnarinone doses.In conclusions, quinolinone derivatevesnarinone had a strong antitumor activity against oral malignant Burkitt lymphoma cells.

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Introduction

Burkitt's lymphoma (BL) is a high-grade B-cell neoplasm and is one of the most aggressive malignancies of lymphoid origins, and accounts for 3-5% of all lymphomas. Generally, BL is found in the pediatric population and it represents 40% of childhood non-Hodgkin lymphoma.[1] The highest incidence is found in the endemic form of equatorial regions of Africa and Papua-New Guinea where it accounts for 50-70% of all pediatric malignancies.[2] BL is characterized by chromosome translocations between the proto-oncogene C-MYC and one of the immunoglobulin (Ig) loci.[3] It was reported Epstein-Barr virus (EBV) has been implicated in its etiology and increased the aggressive of BL. BL most often involves the maxilla or the mandible in oral cavity.[4] Treatment for this type of tumor is still limited. However, the development of a more effective therapeutic method of oral malignant Burkitt's lymphoma must continue. Interestingly, study on malignant Burkitt's lymphoma is still unclear and little reported in Dentistry.

3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone vesnarinone is a newly synthesized positive oral inotropic agent who has been used for the treatment of chronic heart failure.[5] Vesnarinone has multiple biological activities on mammalian cells both *in vitro* and *in vivo*. The mechanisms of action associated with the inotropic properties include a decrease in potassium efflux with an increase in the inward calcium current[6] and an inhibition of

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phosphodiesterase activity.[7] Vesnarinone also has an immunomodulation effect by inhibiting the production of various cytokines, including TNF-α, IFN-γ, IL-1α, IL-2 and IL-6 in polysaccharide-stimulating peripheral blood mononuclear cells,[8] and is shown to inhibit the production of HIV-1 in culture.[9] Vesnarinone was reported in relation to its antitumor effect with apoptosis-inducing activity.[6,8] Also, it has been found to suppress the growth of a wide variety of tumor cell line including human gastric cancer,[9] lung cancer,[10] hepatocelullar carcinoma,[11] salivary gland carcinoma,[12] acute myeloid leukemia,[13] and pancreatic cancer.[14]

In the present study, the antitumor activity of chronic heart failure drug vesnarinone against oral malignant Burkitt lymphoma (Raji) cells through analysis of proliferative barriers, migratory chemotactic inhibition and cell apoptosis inductionwere investigated.

Materials and methods

Cell and cell culture

Raji cell (ATCC CCL-86) was obtained from Department of Paracytology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Cell line was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, and 100mg/ml streptomycin, 100 U/ml penicillin (Moregate BioTech, Bulimba, Australia). The cultures were incubated in humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Drugs dilution

A stock solution of vesnarinone (OPC-8212) with 1.25×10^{-1}M concentration was diluted into 1.25×10^{-2}, 2.5×10^{-2} and 5×10^{-2} M in a solution of DMEM 10% FBS. These concentrations were incubated with Raji cells for 24 and 48 hours.

Cell growth suppression (MTT assay)

Raji cells were seeded on 96-well plates (Falcon, NJ, USA) at 2.0×10^{4} cells per well in DMEM containing 10% FCS, the day before treatment. Cell line was treated with various doses of vesnarinone [0, 1.25×10^{-2}, 2.5×10^{-2} and 5×10^{-2} M, and IC_{50} carboplatin (3.1×10^{-6} M) as positive control]. After 24 and 48 hours, the number of cells was quantitated by an assay in which MTT;3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazoliumbromide (Sigma-aldrich) was used.[15] Measurement of absorbance was detected by BioRad microplate reader (BioRad, USA) with wave length 540 nm.

Chemotactic migration assay using Boyden chamber kit

Chemotaxis (directed migration) was evaluated in the Boyden chamber apparatus (Neuro Probe, Inc., Cabin John, MD, USA). Briefly, sub confluent cells were starved for 24 h and harvested with 0.05% (w/v) trypsin (Invitrogen Corporation, USA) containing 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA, Invitrogen Corporation). Thus, washed twice with PBS, and resuspended to a final concentration of 5×10^{5} per ml in serum-free medium with 0.1% (w/v) fraction V bovine serum albumin (BSA, Wako Pure Chemical Industries, Ltd). Polyvinyl-pyrrolidone (PVP) filters (Nuclepore Corp, Palo Alto, CA, USA) of 8-μm pore size were precoated with gelatin (Merck KGaA, Frankfurt, Darmstadt, Germany) (0.1 mg/ml) and rinsed in sterile water. Lower chamber was filled with 30 μl of 10% FBS in DMEM plus various doses of vesnarinone and covered with a gelatin-coated membrane. Furthermore, 50 μl of cell suspension, yielding 500 cells/ml of Raji cells were added to the upper chamber. After 24 hours of incubation, the membrane was stained with Giemsa solution (Ted Pella Inc., Redding, CA, USA). The number of cells had penetrated through the filter was counted under light microscope at 400x magnification. The counting was performed for 12 fields in each concentration.[15]

Induction of apoptosis using double-staining (AO-EB) analysis

Acridine orange (AO) and ethidium bromide (EB) double staining were carried out in this study. DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with

different doses of vesnarinone for 24 h, the cells were detached, washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 min. The stained cells were observed by a fluorescence microscope (Zeiss, Germany) at 100 × magnifications. The cells were divided into three categories as follows: viable cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation). In each experiment more than 100 cells/sample were counted.

Statistical analysis

Statistical differences between the means of the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using two-way ANOVA followed by a post-hoc *t-test*. The significance level was set at 5% for each analysis.

Results

Cell growth inhibition (MTT assay)

Raji cells treated with various doses of vesnarinone were examined by the MTT assay. Relative cell number was evaluated by comparing the absorbance in each group. No significant differences in cell number were observed in concentration of 1.25×10^{-2} and 2.5×10^{-2} M, but in concentration of 5×10^{-2} M and positive control were found a significant difference of cell growth compared with that of negative control in 24 h. Furthermore, the cell growth of Raji cells treated with concentration 2.5×10^{-2} M, 5×10^{-2} M and positive control were significantly suppressed in 48 h as compared to control cells (P<0.05). Increased of cell growth inhibition in 5×10^{-2} M at 24 h was found at 36.65%, while at 48 h of 65.20% (Figure 1).

In vitro chemotactic migration assay

Cell migration is a main process involved in tumor invasion and metastasis. The ability of cell migration on each treated cell with the Boyden chamber kit was evaluated for 24 hours incubation. As seen in figure 2, Raji-treated cells with 2.5×10^{-2} and 5×10^{-2} Mof vesnarinone were markedly showed the low ability of cell migration compared with that of control (P<0.05; one-way ANOVA). Raji cells treated with 5×10^{-2} Mhad the potential barriers to migration at 73.8% and IC_{50} carboplatin (3.1×10^{-6} M) at 77.3%.

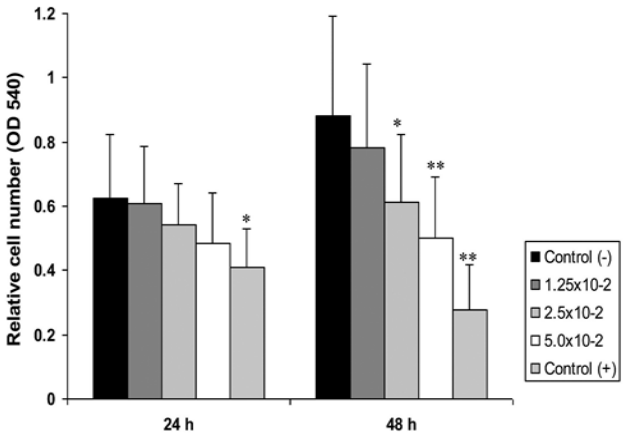

Figure 1. Relative Cell Number of Raji Cell Incubated with Various Doses of Vesnarinone (unit: M) which Observed by MTT Assay for 24 and 48 hours (*P<0.05).

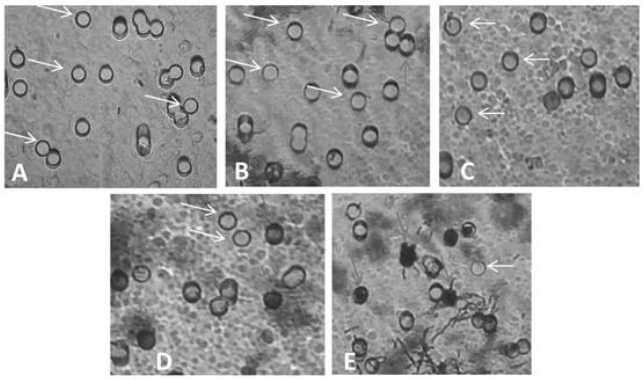

Figure 2. Chemotactic Migration of Raji Cells were Treated by Different Doses of Vesnarinone. A). Negative Control, B). dose of $1.25x10^{-2}$ M., C). dose of $2.5x10^{-2}$ M., D). dose of $5x10^{-2}$ M., E. IC_{50} carboplatin dose 3.1×10^{-6} M (White Arrows: of Viable Cells, Red Arrow: Cell Apoptosis).

Induction of apoptosis using double-staining (AO-EB) analysis

The induction of cell apoptosis treated with vesnarinone has been observed *in vitro* on oral malignant Burkitt's lymphoma cells through a double staining analysis of Acridine orange-Ethidium bromide. The result revealed vesnarinone was markedly increased the apoptosis of Raji cell compared with that of negative control. Increased cell apoptosis was also occured in Raji cells induced by carboplatin IC_{50} (positive control)

(Figure 3). Based on Figure 3, viable cells were appeared the green color, while the apoptotic cells were showed the yellow-orange color. Raji cells treated with vesnarinone show an increase in apoptotic activity characterized by the number of cells with yellow-orange colors. In the present study, the highest dose of vesnarinone had high apoptotic activity on the Raji cells. It was also found that positive control had high apoptotic activity against Raji cells.

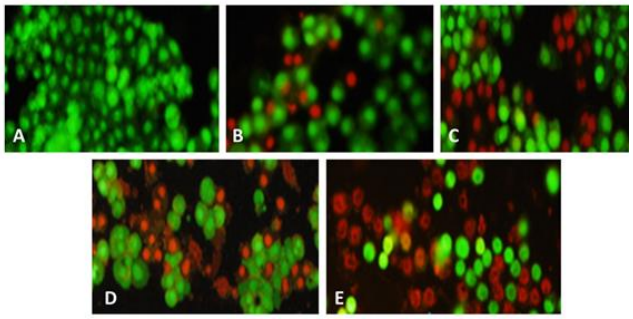

Figure 3. Apoptosis of Raji cells were treated with various doses of vesnarinone. A). negative control, B). dose $1.25x10^{-2}$ M., C). dose 2.5×10^{-2} M., D). dose 5×10^{-2} M., E. positive control of carboplatin IC_{50} (3.1×10^{-6} M). (Green color was viable cell, and yellow-orange color was apoptotic cells).

Discussion

Balancing between positive and negative regulatory factors is required for the cell cycle. Any alteration in this condition can result in abnormal cell proliferation, which may contribute to cancer. If cancer has occurred, then the treatment becomes more difficult and complex. During this period, increased growth, invasion and metastasis suppression and apoptosis induction of cells through targeted therapies have become the attention center of researchers in the field of oral cancer.[15] It requires continuous research to find the effective methods and potential drug agents against oral cancer cells. In the present study, the antitumor activity of chronic heart failure drug vesnarinone against oral malignant Burkitt lymphoma (Raji) cell through analysis of proliferative barrier, migratory chemotactic inhibition and induction of cell apoptosis was examined.

Our results suggest vesnarinone showed to be a potent therapeutic agent for oral malignant Burkitt's lymphoma cells through increased cell proliferation inhibition and low ability of migratory chemotactic. It was found that the inhibition of cell proliferation and migration treated with vesnarinone dose 5×10^{-2} M was 65.2% to 73.8%. These results suggest that vesnarinone had a strong activity in suppressing Raji cell proliferation, although the suppression of these cells was not as strong as positive control. This data revealed that IC_{50} of carboplatin used as a positive control is very toxic to Raji cells or still too high dose (Carboplatin IC_{50} dose used in this study was taken from IC_{50} of head, colon, breast and uterine cancer). The most important result was an increase in cell proliferation and migratory chemotactic inhibition occured in Raji cells treated with vesnarinone followed by an increase in cell apoptosis. These results may involve a variety of complex protein-blocking mechanisms including protein cyclin-dependent kinase inhibitor, cell-cycle arrest, matric metallo protein (MMP), Akt/PKB transduction signal proteins, NF-kB transcription factor protein, and induction of pro-aptoptosis protein. Honma et al.[10] reported that vesnarinone has the potential to increase cell cycle arrest in G1 phase in lung carcinoma cells. Vesnarinone also causes cell cycle inhibition in G0-G1 phase with decreased expression of cyclin A, D, E and cyclin-dependent kinase-2 (CDK-2) expression in gastric cancer.[9] Decreased expression of cell cycle proteins is known to activate the cyclin-dependent kinase inhibitor protein p27Kip1 as a negative regulator of cell cycle that can increase cell growth restriction and apoptotic induction in oral squamous cell carcinoma and salivary gland cancer.[16,17] It has also been reported that vesnarinone showed the ability to inhibit angiogenesis and tumorigenisity in oral squamous cell carcinoma through expression barrier of VEGF growth factor protein and family of IL-8 cytokines.[16] Furthermore, vesnarinone may inhibit the activity of transcription factor AP-1, TNF-α, NF-κB, c-Jun protein and increase apoptosis of human cancer cells according to doses and drug dependent time.[18] Our results showed the greater dose of vesnarinone appeared the low activity of migratory chemotactic cells and inhibition of cell proliferation at 24 and 48 hour. In contrast, apoptotic cells increased with higher vesnarinone doses, although the number of cell apoptosis is not as strong as positive controls.

An increase in activation of apoptosis characterized by induction of yellow-orange color

cells in the various doses of vesnarinone-treated cells strongly suggested that apoptosis had occurred in those cultures. An antitumor effect of vesnarinone was reported by an up-regulation of p27 and down-regulation of Jab1, VEGF, IL-8, NF-kB and TNF-α.[16,18] Also, p53 expression was increased.[19] Recent study reported that p53 and 16 played a role in oral potentially malignant disorder.[20] As expected from its potency in cell proliferation and migratory chemotactic barriers and apoptosis induction, a marked suppression of vesnarinone was detected in dose 2.5×10^{-2} M and 5×10^{-2} M.

The use of carboplatin drug (the one of the platinum families) as a positive control in this study was intended because platinum group is the drug of choice for therapy of oral squamous cell carcinoma.[21] Carboplatin as a major chemotherapy drug is known to be effective against several types of human cancers including carcinoma, sarcoma, germ cell tumor, Hodkin's lymphoma and Non-Hodkin's lymphoma.[22] The therapeutic effect of carboplatin will be more potent and adequat when combined with other chemotherapeutic drugs such as 5-FU, doxorubicin, docetaxel, vincristine and others.[16] In our study, IC_{50} carboplatin had a stronger antitumor activity against Raji cells than dose 5×10^{-2} M vesnarinone.

Conclusions

Based on the results of study can be concluded 3,4-dihydro-6-[3,4-dimethoxybenzoyl -1-piperazinyl]-2(1H)-quinolinone vesnarinone had a strong antitumor activity against oral malignant Burkitt lymphoma cells through analysis of proliferative barriers, migratory chemotactic suppression and cell apoptosis induction.

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Declaration of Interest

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